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ArtinM, a D-mannose-binding lectin from *Artocarpus integrifolia*, plays a potent adjuvant and immunostimulatory role in immunization against *Neospora caninum*

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ABSTRACT

ArtinM and Jacalin (JAC) are lectins from the jackfruit (*Artocarpus integrifolia*) that have important role in modulation of immune responses to pathogens. *Neospora caninum* is an Apicomplexa parasite that causes neuromuscular disease in dogs and reproductive disorders in cattle, with economic impact on the livestock industry. Hence, we evaluated the adjuvant effect of ArtinM and JAC in immunization of mice against neosporosis. Six C57BL/6 mouse groups were subcutaneously immunized three times at 2-week intervals with *Neospora* lysate antigen (NLA) associated with lectins (NLA + ArtinM and NLA + JAC), NLA, ArtinM and JAC alone, and PBS (infection control). Animals were challenged with lethal dose of Nc-1 isolate and evaluated for morbidity, mortality, specific antibody response, cytokine production by spleen cells, brain parasite burden and inflammation. Our results demonstrated that ArtinM was able to increase NLA immunogenicity, inducing the highest levels of specific total IgG and IgG2a/IgG1 ratio, ex vivo Th1 cytokine production, increased survival, the lowest brain parasite burden, along with the highest inflammation scores. In contrast, NLA + JAC immunized group showed intermediate survival, the highest brain parasite burden and the lowest inflammation scores. In conclusion, ArtinM presents stronger immunostimulatory and adjuvant effect than Jacalin in immunization of mice against neosporosis, by inducing a protective Th1-biased pro-inflammatory immune response and higher protection after parasite challenge.

1. Introduction

Neospora caninum is an Apicomplexa protozoan parasite that was described in 1988 and first identified in dogs causing neuromuscular disease [1]. The veterinary importance of *N. caninum* became known a few years later its discovery, when it was found to cause abortion and reproductive disorders in cattle worldwide, leading to considerable economic losses [2]. Currently, *N. caninum* is recognized to infect naturally and experimentally a wide range of intermediate hosts, including domestic and sylvatic animals [3]. The herbivorous intermediate hosts as cattle acquire infection horizontally by ingestion of oocysts excreted by canine definitive hosts, and often vertically during pregnancy, likely due to the imbalance of the immune system by fetal regulatory cytokines, such as IL-10 and IL-4, leading to recrudescence and differentiation of tissue cyst-contained bradyzoites into tachyzoites with subsequent parasitemia [4]. Afterward, parasites may cross the placenta and infect the fetus, causing abortion or congenital infection, depending on the gestation period and the time of infection [5]. Immune response to *N. caninum* is known to be predominantly of the Th1-type, with involvement of $CD4^+$ T cells, production of IL-12 and IFN-γ, whereas B cells and antibodies have been considered important for controlling the spread of parasite extracellular stages [6]. Also, innate immunity participates in protective mechanisms against neosporosis, involving the recognition of conserved pathogen-associated molecular patterns by Toll-like receptors (TLRs) [7].

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Protein–carbohydrate recognition is crucial to diverse intracellular processes, such as interactions among different cells or cells and extracellular matrix, cell adhesion and migration, embryogenesis, and development of immune responses, since it can be the initiator of a functional crosstalk that modulates their physiology and homeostatic balance [8]. In this context, lectins are proteins with capacity to bind specifically to carbohydrates and can be isolated from many different sources, including plant and animal tissues [9]. Several plant lectins with interesting biological properties have been prepared from the Moraceae family, including Jacalin and ArtinM from seeds of jackfruit (*Artocarpus integrifolia*) [10,11]. Structural differences account for the distinct carbohydrate binding specificities exhibited by Jacalin and ArtinM, the latter previously known as KM^+ or Artocarpin [12]. Whereas ArtinM binds to a wide range of monosaccharides, with preferential affinity for mannose [11], Jacalin, the major protein from *A. integrifolia* seeds, preferentially binds to the disaccharide Galβ1-3GalNAc, being also characterized as an IgA- and IgD-binding lectin [10,13]. Although ArtinM and Jacalin have been described with regards to their immunostimulatory role on the innate immune system, as well as their adjuvant effects in murine models of immunization against protozoan parasites as *Trypanosoma cruzi* [14] and *Leishmania* spp [15,16], their use has not yet been investigated for neosporosis.

Among the control and prevention measures of neosporosis, the development of effective vaccines presents interesting challenges, with the use of murine models to characterize novel antigens and strategies for successful vaccination [17]. A wide range of approaches has been evaluated, including live or inactivated vaccines [18–22], subunit or recombinant vaccines using a number of parasite surface proteins [23–26], and recombinant virus vector vaccines [27]. All these strategies have shown that protection is sometimes partial and depends on the type of antigen and adjuvant used, as well the delivery systems. For this reason, we evaluated in the present study the role of the lectins ArtinM and Jacalin as adjuvants in immunization of mice against *N. caninum* infection associated or not with *Neospora* lysate antigen.

2. Material and methods

2.1. Parasite and antigens

N. caninum tachyzoites (Nc-1 isolate) [28] were maintained by serial passages in Vero cell line cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2% heat-inactivated calf fetal serum (CFS) at 37 °C in a 5% CO_2 atmosphere. Parasite suspensions were obtained as previously described [29]. Briefly, tachyzoites were harvested by scraping off the cell monolayer after 48–72 h of infection, passed through a 26-gauge needle to lyse any remaining intact host cell, and centrifuged at low speed (45 × *g*) for 1 min at 4 °C to remove host cell debris. The supernatant containing parasite suspension was collected, washed twice (700 × *g*, 10 min, 4 °C) in phosphate-buffered saline (PBS, pH 7.2) and the resulting pellet was resuspended in PBS. Parasites were counted in hemocytometric chamber using 0.4% Trypan blue vital staining and stored at −20 °C until antigen preparation or immediately used for challenge of immunized animals.

Neospora lysate antigen (NLA) was prepared as described elsewhere [29]. Parasite suspension (1×10^8 tachyzoites/ml) was treated with protease inhibitors (1.6 mM PMSF, 50 μg/ml leupeptin and 10 μg/ml aprotinin) and lysed by ten freeze–thaw cycles followed by ultrasound on ice. After centrifugation (10,000 × *g*, 30 min, 4 °C), supernatant was collected, filtered in 0.22 μm membranes and its protein concentration determined by bicinchoninic acid (BCA) assay [30]. NLA aliquots were stored at −70 °C until their use in immunization of mice, serological tests and cytokine production assays.

N. caninum tachyzoites were also prepared for using in indirect fluorescent antibody test (IFAT) as previously described [29]. Parasite suspension (1×10^6 tachyzoites/ml) was treated with 1% formaldehyde for 30 min at room temperature. After washing twice in PBS, parasites were dry-fixed in microscopic slides and stored at −20 °C.

2.2. Lectins ArtinM and Jacalin from A. integrifolia

ArtinM and Jacalin from *A. integrifolia* were prepared in one of our laboratories (MCRB). The total extract preparation of seeds from *A. integrifolia*, as well as their purification to generate D-mannose (ArtinM)- and D-galactose (Jacalin)-binding lectins, were performed as previously described [11,13]. The homogeneity and purity degree of the lectins were evaluated by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE at 15%) under non-reducing conditions.

2.3. Animals and immunization

All experiments were carried out with 8–12-week-old female C57BL/6 mice maintained under standard conditions in the Bioterism Center and Animal Experimentation, Federal University of Uberlândia, MG, Brazil. All procedures were conducted according to guidelines for animal ethics and the study received approval of the Ethics Committee for Animal Experimentation of the institution.

Six groups of 13 mice were immunized subcutaneously (200 μl/animal) three times at two-week intervals, as follows: 25 μg NLA mixed with 1 μg ArtinM in sterile PBS (NLA + ArtinM group); 25 μg NLA mixed with 100 μg Jacalin in sterile PBS (NLA + JAC group); 25 μg NLA alone (NLA group); 1 μg ArtinM alone (ArtinM group); 100 μg Jacalin alone (JAC group); and diluent only (PBS group). The adopted doses of antigen and lectins were based on previous studies [14,15,29]. Blood samples were collected at 0, 15, 30, 45 and 60 days after immunization (d.a.i.), and the sera stored at −20 °C until to be analyzed for the presence of specific antibodies.

2.4. Determination of N. caninum-specific total IgG, IgG1 and IgG2a antibodies

Levels of *N. caninum*-specific total IgG, IgG1 and IgG2a antibodies were measured by ELISA as described elsewhere [29], with modifications. High-affinity microtiter plates were coated with NLA (10 μg/ml), washed with PBS plus 0.05% Tween 20 (PBS-T) and blocked with 5% skim milk in PBS-T for 1 h at room temperature. Serum samples were diluted 1:25 in 1% skim milk-PBS-T and incubated for 1 h (for IgG detection) or 2 h (for IgG1 and IgG2a detection) at 37 °C. After washing, peroxidase-labeled goat anti-mouse IgG (1:1000; Sigma Chemical Co., St Louis, MO) or biotin-labeled goat anti-mouse IgG1 (1:4000) or anti-mouse IgG2a (1:2000) antibodies (Caltag Lab. Inc., South San Francisco, CA) were added and incubated for 1 h at 37 °C. Next, streptavidin-peroxidase (1:1000; Sigma) was added for IgG1 and IgG2a detection assays. The assays were developed with 0.01 M 2,2-azino-bis-3-ethyl-benzthiazoline sulfonic acid (ABTS; Sigma) and 0.03% H_2O_2. Optical density (OD) values were determined in a plate reader at 405 nm. Results were expressed in ELISA index (EI) as previously described [31], according to the formula: EI = OD sample/OD cut off, where cut off was calculated as the mean OD for negative control sera plus three standard deviations.

2.5. Immunostaining of N. caninum tachyzoites

To verify *N. caninum* immunostaining, IFAT was performed with mouse sera collected at 45 d.a.i. as previously described [29]. Slides containing formolized tachyzoites were incubated with serum samples diluted 1:50, and then with FITC-labeled goat anti-mouse IgG (1:50; Sigma). Slides were overlaid with buffered glycerol and examined in fluorescence microscope (EVOS, Advanced Microscopy Group, Inc., Mill Creek, WA).

2.6. Cytokine production

Two weeks after the last immunization (45 d.a.i.), three mice from each group were euthanized and their spleens were aseptically removed for cell culture and cytokine production assay. Mouse spleens were dissociated in RPMI medium and cell suspensions were washed in medium, treated with lysis buffer (0.16 M NH_4Cl and 0.17 M Tris–HCl, pH 7.5), washed again and resuspended in complete RPMI medium containing 10% CFS. Viable cells (2×10^5 cells/200 μl/well) were cultured in triplicate in 96-well plates in the presence of antigen (NLA, 10 μg/ml), mitogen (Concanavalin A – ConA, 2.5 μg/ml) or medium alone and incubated at 37 °C in 5% CO_2. After 48 h, cell-free supernatants were collected and stored at −70 °C for cytokine quantification. IL-10 and IFN-γ measurements were carried out by sandwich ELISAs according to manufacturer's instructions (R&D Systems, Minneapolis, MN). The limit of detection for each assay was 31 pg/ml and intra-assay variation coefficients were below 15%.

2.7. Challenge

After 30 days of the last immunization (60 d.a.i.), the remaining animals of each group (10 per group) were challenged intraperitoneally (200 μl/mouse) with 2×10^7 low-passage Nc-1 tachyzoites. Animals were observed daily for clinical signs through morbidity scores, body weight changes and mortality during 30 days post-infection (d.p.i.). Morbidity scores were calculated as described elsewhere [32], with minor modifications as follows: sleek/glossy coat, bright and active (score 0); ruffled coat (score 1); hunched, tottering gait, starry stiff coat (score 2), reluctance to move (score 3). Results were expressed as the mean of the scores given daily to each animal for each group. After 30 days of challenge, surviving animals were euthanized and blood samples and brain tissues were collected. Serum samples were tested for *N. caninum* serology and brain tissues were sliced longitudinally, being half of them stored at −70 °C for polymerase chain reaction (PCR) assay. The remaining tissue was fixed in 10% buffered formalin, embedded in paraffin and routinely processed for immunohistochemical and histological assays.

2.8. Determination of parasite burden and inflammatory scores

Brain parasite load was determined by quantitative real-time PCR as previously described [29], using primer pairs (sense 3′ GCTGAACACCGTATGTCGTAAA-5′; antisense 3′-AGAGGAATGCCACATAGAAGC-5′) to detect the *N. caninum* Nc-5 sequence through SYBR green detection system (Invitrogen, San Francisco, CA). DNA extraction was performed from 20 mg of murine brain tissues (Genomic DNA kit, Promega Co., Madison, WI) and parasite loads were calculated by interpolation from a standard curve from Nc-1 tachyzoite DNA included in each run (7500 Real time PCR System, Applied Biosystems, Foster City, CA). As negative control, brain tissue from non-immunized and unchallenged mice was analyzed in parallel.

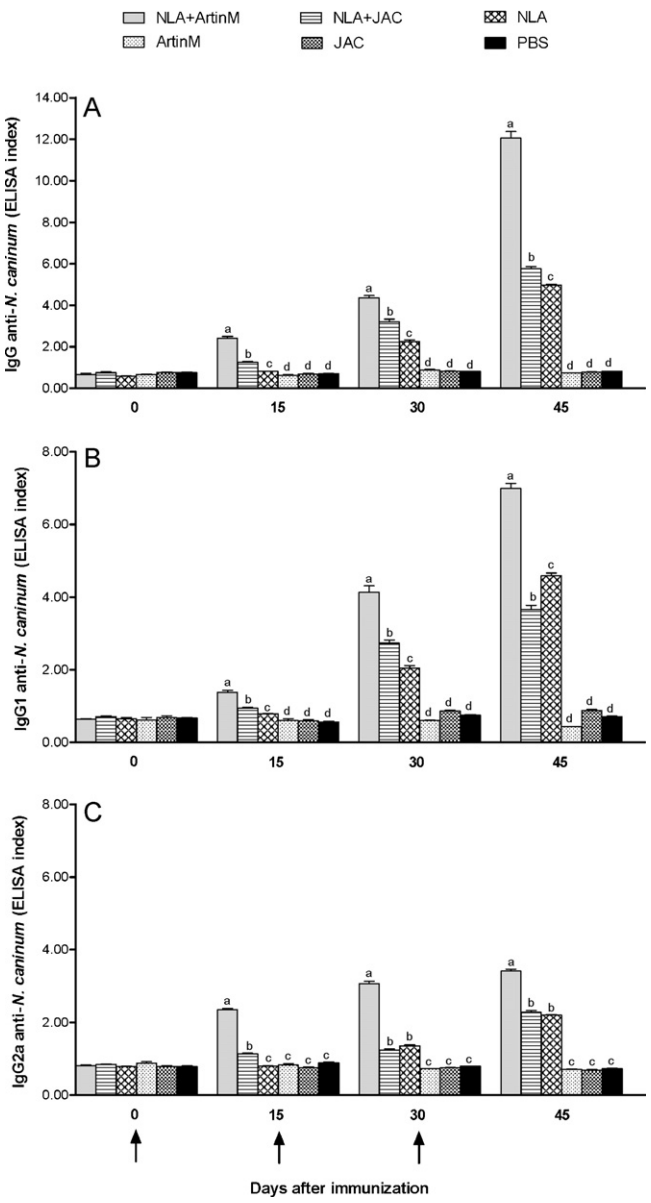

Fig. 1. Levels of total IgG (A), IgG1 (B) and IgG2a (C) anti-*N. caninum* determined by ELISA in serum samples of C57BL/6 mice. Six mouse groups (13 animals per group) were immunized subcutaneously three times (black arrows) with *Neospora* lysate antigen (NLA) associated with lectins from *A. integrifolia*, ArtinM (NLA + ArtinM) or Jacalin (NLA + JAC). As controls, mice were inoculated with NLA alone (antigen control), ArtinM or JAC alone (lectin controls) or PBS (infection control). Blood samples were collected at 0, 15, 30, and 45 days after immunization. Values are indicated as ELISA index and expressed as mean ± SEM of two independent experiments. [a–d]Different letters indicate statistically significant differences among the groups in each time point analyzed ($P < 0.05$; ANOVA and Bonferroni multiple comparison post-test).

Brain tissue parasitism was also determined by immunohistochemistry as previously described [29]. Briefly, deparaffinized sections were blocked with 3% H_2O_2 and treated with 0.2 M citrate buffer (pH 6.0) in microwave oven to rescue antigenic sites. Next, sections were blocked with 2% non-immune goat serum and subsequently incubated with primary antibody (pooled sera from mice experimentally infected with *N. caninum*), secondary biotinylated goat anti-mouse IgG antibody (Sigma) and avidin–biotin complex (ABC kit, PK-4000; Vector Laboratories Inc., Burlingame, CA). The reaction was developed with 0.03% H_2O_2 plus 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) and slides

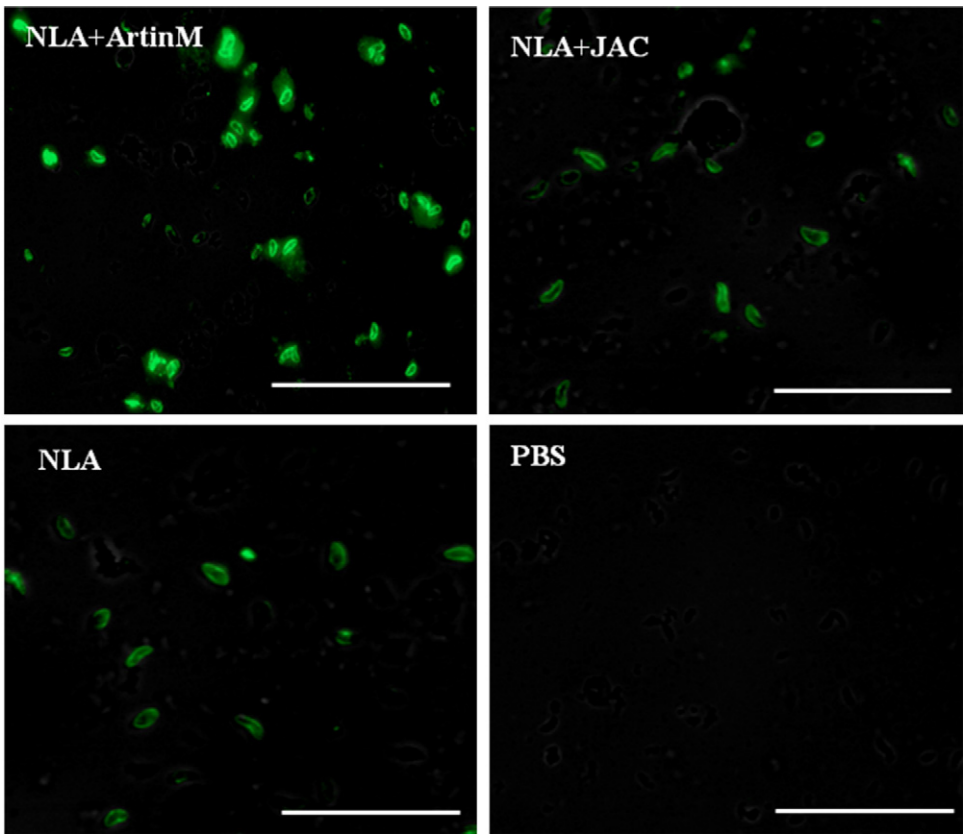

Fig. 2. Immunostaining of *N. caninum* tachyzoites. C57BL/6 mice were immunized with *Neospora* lysate antigen (NLA) associated with lectins from *A. integrifolia*, ArtinM (NLA + ArtinM) or Jacalin (NLA + JAC), or NLA alone (antigen control), or PBS (infection control). Sera were analyzed at 45 days after immunization by indirect fluorescent antibody test. Bar scale: 100 μm.

were counterstained with Harris haematoxylin until to be examined under light microscopy. Tissue parasitism was evaluated by counting the number of free parasites and parasitophorous vacuoles in 160 microscopic fields in at least four mouse tissue sections for each group.

Histological changes were analyzed in two cerebral noncontiguous sections (40 μm distance between them) stained with haematoxylin and eosin obtained from each mouse and from at least four mice per group [33]. The inflammatory score was represented as arbitrary units: 0–1, mild; 1–2, moderate; 2–3, severe and >3, very severe. Negative controls included cerebral tissue from non-immunized and unchallenged mice. All analyses were done in a magnification of 1 × 40 in a blind manner by two observers.

2.9. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). The Kaplan–Meier method was applied to estimate the percentage of mice surviving at each time point after challenge and survival curves were compared using the log rank test. Differences between groups were analyzed using ANOVA or Kruskal–Wallis test, when appropriate, with the respective Bonferroni or Dunn multiple comparison post-tests to examine all possible pairwise comparisons. Student *t* test was used for comparison of IgG isotypes and IgG1/IgG2a ratios in different groups. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. N. caninum-specific antibody responses after immunization and challenge

Mice immunized with NLA + ArtinM presented higher total IgG levels to *N. caninum* in comparison to all other groups from 15 to 45 d.a.i. (Fig. 1A). A similar profile was observed with the NLA + JAC group in relation to the remaining groups ($P < 0.05$). Mice immunized with NLA alone showed higher total IgG levels only in relation to control groups (ArtinM, JAC, PBS) from 15 to 45 d.a.i. ($P < 0.05$) (Fig. 1A).

Regarding IgG1 isotype (Fig. 1B), a profile comparable to total IgG was observed from 15 to 30 d.a.i. in all groups, but on day 45 after immunization, IgG1 levels were higher for the NLA group as compared to the NLA + JAC group, even though lower in relation to the NLA + ArtinM group ($P < 0.05$). IgG2a isotype kinetics also showed higher IgG2a levels for the NLA + ArtinM group from 15 to 45 d.a.i. when compared to the other groups, with similar IgG2a levels between NLA + JAC and NLA groups at 30 and 45 d.a.i. (Fig. 1C). All control groups showed IgG, IgG1 and IgG2a levels below the cut off.

N. caninum immunostaining showed a brighter linear peripheral fluorescence of parasite surfaces when probed with sera from mice immunized with NLA + ArtinM in relation to NLA + JAC and NLA groups (Fig. 2). The control group (PBS) showed no staining of tachyzoites.

Serological results determined at 60 days after immunization before challenge (BC) and 30 days after challenge (AC) with 2×10^7

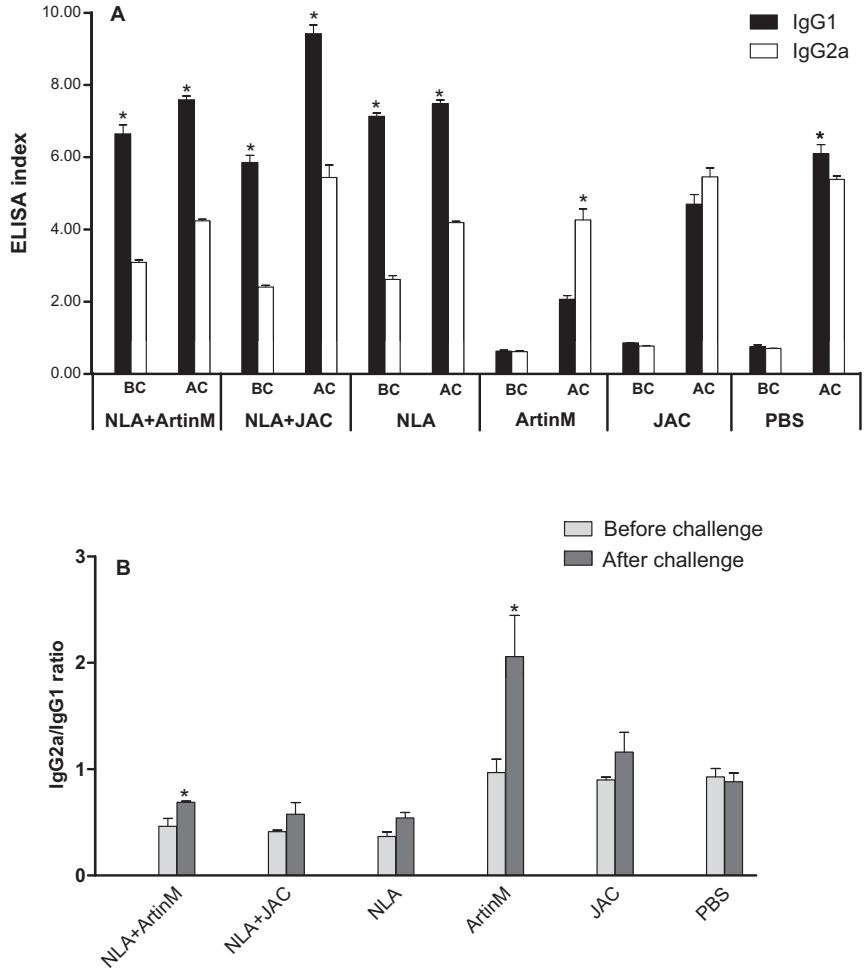

Fig. 3. Comparison between IgG1 and IgG2a responses to *N. caninum* determined by ELISA in serum samples of C57BL/6 mice. Six mouse groups (13 animals per group) were immunized with *Neospora* lysate antigen (NLA) associated with lectins from *A. integrifolia*, ArtinM (NLA + ArtinM) or Jacalin (NLA + JAC). As controls, mice were inoculated with NLA alone (antigen control), ArtinM or JAC alone (lectin controls) or PBS (infection control). (A) Serological results determined at 60 days after immunization before challenge (BC) and 30 days after challenge (AC) with 2×10^7 tachyzoites of Nc-1 isolate. Values are indicated as ELISA index and expressed as mean ± SEM of two independent experiments. (B) IgG2a/IgG1 ratio before and after challenge for each group. Bars represent mean ± SEM. *$P < 0.05$ determined by Student t test when comparing IgG1 and IgG2a levels (A) or before and after challenge (B) in each group.

tachyzoites of Nc-1 isolate. *N. caninum*-specific IgG1 and IgG2a isotypes were compared before challenge (60 d.a.i.) and 30 days after challenge (90 d.a.i.) with virulent parasite in all experimental groups, including the assay of seroconversion for the control groups (Fig. 3A). Levels of IgG1 were higher than IgG2a in all antigen-immunized groups regardless of the lectin adjuvant in both conditions, before and after parasite challenge, while a seroconversion with predominant IgG2a response was observed after parasite challenge only in the lectin-immunized groups, but with significant difference for ArtinM lectin alone ($P < 0.05$). PBS group showed seroconversion with no significant difference between IgG1 and IgG2a isotypes after challenge (Fig. 3A). It was also observed an increase of the IgG2a/IgG1 ratio after challenge in all groups immunized with antigen and/or lectin, although with significant increase only in the NLA + ArtinM and ArtinM groups ($P < 0.05$) (Fig. 3B).

3.2. Cytokine production after immunization

Ex vivo cytokine production was assessed in spleen cell cultures at 45 d.a.i. and supernatants of these cells were collected after 48 h of stimulation with medium, ConA or NLA (Fig. 4A and B). After antigen stimulation, IFN-γ levels were higher in the NLA + ArtinM group in relation to all others ($P < 0.05$) (Fig. 4A). ConA stimulation induced increased levels of IFN-γ in all groups in relation to baseline (medium), particularly when mice were immunized with NLA alone (Fig. 4A).

Increased levels of IL-10 were detected in both NLA + ArtinM and NLA groups as compared with other groups after antigen stimulation ($P < 0.05$), whereas NLA + JAC group showed higher IL-10 levels in relation to the controls only ($P < 0.05$) (Fig. 4B). In all groups, mitogenic stimulation induced increased IL-10 levels compared to baseline, but with lower levels in relation to antigenic stimulation, mainly in antigen-immunized groups. As shown in Fig. 4C, mice immunized with NLA + ArtinM showed the highest IFN-γ/IL-10 ratio followed by the ArtinM group ($P < 0.05$), whereas the NLA + JAC and NLA groups exhibited the lowest IFN-γ/IL-10 ratio ($P < 0.05$).

3.3. Protection after parasite challenge

Protection after Nc-1 parasite challenge was evaluated by clinical parameters as morbidity scores, body weight changes from baseline and survival (Fig. 5). Mice immunized with NLA + ArtinM or ArtinM alone presented the highest scores of morbidity (Fig. 5A) and the most pronounced body weight losses (Fig. 5B) in relation to other groups ($P < 0.05$). In contrast, NLA + JAC and NLA groups showed the lowest scores of morbidity (Fig. 5A) ($P < 0.05$), with no significant weight changes. JAC and PBS groups also showed no

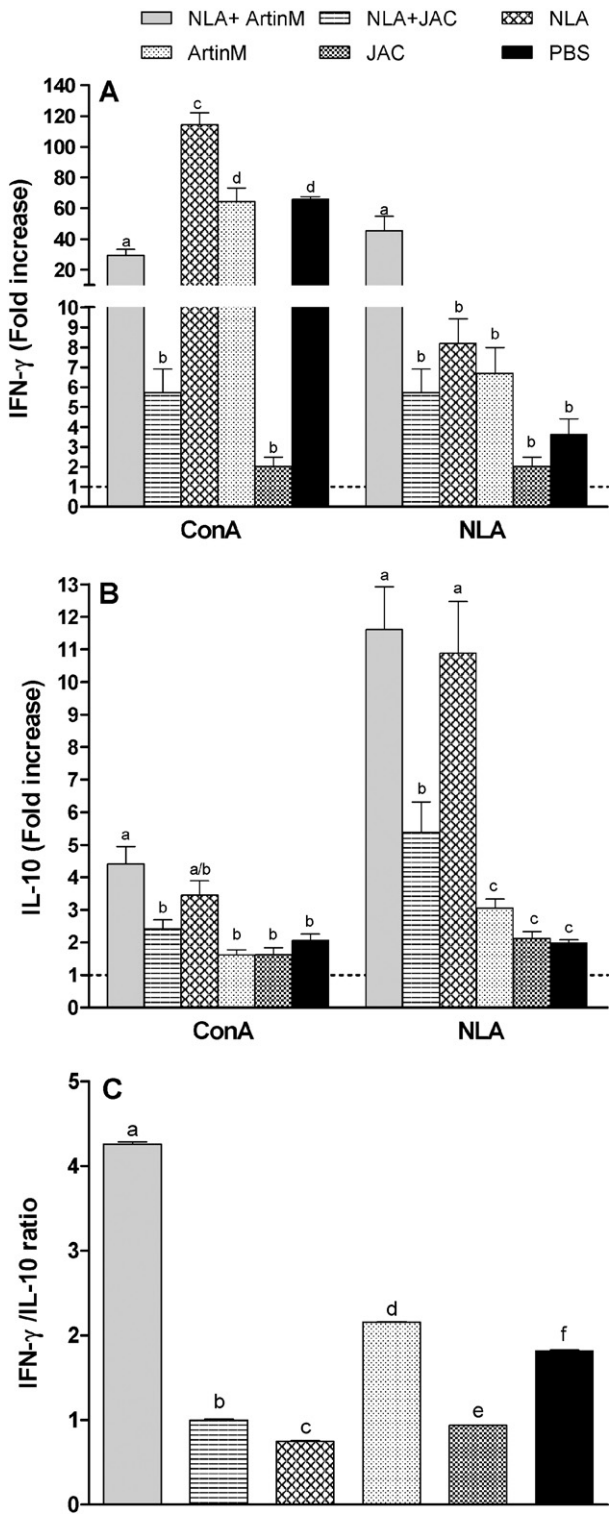

Fig. 4. Cytokine production of spleen cells from mice immunized with *Neospora* lysate antigen (NLA) associated with lectins from *A. integrifolia*, ArtinM (NLA + ArtinM) or Jacalin (NLA + JAC). As controls, mice were inoculated with NLA alone (antigen control), ArtinM or JAC alone (lectin controls) or PBS (infection control). Spleen was collected from three mice per group at 45 days after immunization and cells were cultured in the presence of mitogen (Concanavalin A [ConA] 2.5 μg/ml), antigen (NLA, 10 μg/ml) or medium alone. Supernatants were collected after 48 h and analyzed for IFN-γ (A) and IL-10 (B) by sandwich ELISAs. The IFN-γ/IL-10 ratio was calculated only for NLA stimulation (C). Values are indicated as mean ± SEM of cytokine levels in relation to baseline (medium) of two independent experiments. The dashed line represents the baseline. [a–f]Different letters indicate statistically significant differences among the groups in each time point analyzed (*$P < 0.05$; ANOVA and Bonferroni multiple comparison post-test).

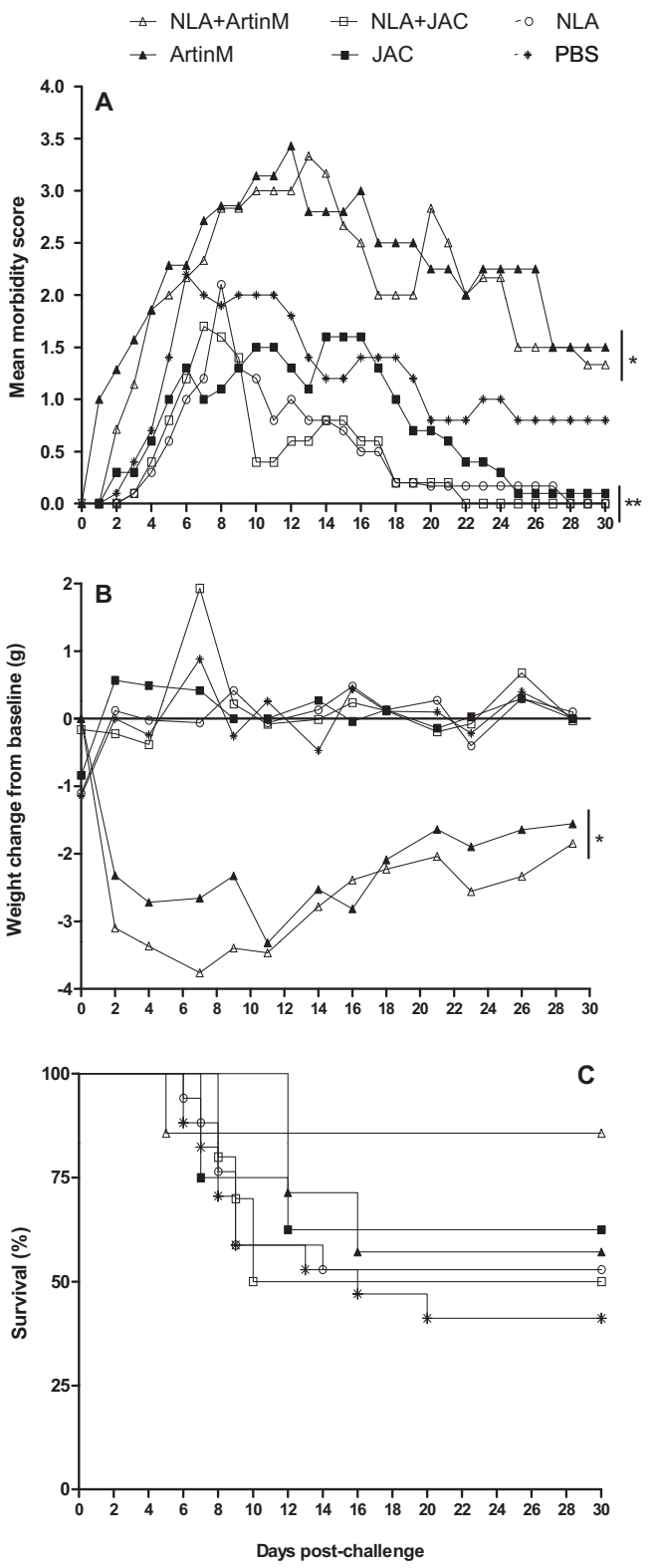

Fig. 5. Mean morbidity score (A), body weight change from baseline (B) and survival curves (C) of C57BL/6 mice after challenge with *N. caninum*. Six mouse groups (13 animals per group) were immunized with *Neospora* lysate antigen (NLA) associated with lectins from *A. integrifolia*, ArtinM (NLA + ArtinM) or Jacalin (NLA + JAC). As controls, mice were inoculated with NLA alone (antigen control), ArtinM or JAC alone (lectin controls) or PBS (infection control). Mice (10 animals per group) were challenged with 2×10^7 tachyzoites of Nc-1 isolate. Values are representative of two independent experiments. *$P < 0.05$ when comparing NLA + ArtinM and ArtinM groups with other groups; **$P < 0.05$ when comparing NLA + JAC and NLA groups with other groups (ANOVA and Bonferroni multiple comparison post-test).

significant weight changes and morbidity scores. Regarding the survival curves (Fig. 5C), the highest survival rate (86%) was observed for NLA + ArtinM group, whereas the PBS control group had the lowest survival (41%) ($P < 0.05$). Mice immunized with NLA + JAC, NLA, ArtinM or JAC presented intermediate survival rates (50–62%) (Fig. 5C).

3.4. Brain parasite burden and inflammation after parasite challenge

Brain parasite burden after Nc-1 challenge determined by real-time PCR (Fig. 6A) was lower in mice immunized with NLA + ArtinM and ArtinM alone than in NLA + JAC and PBS groups

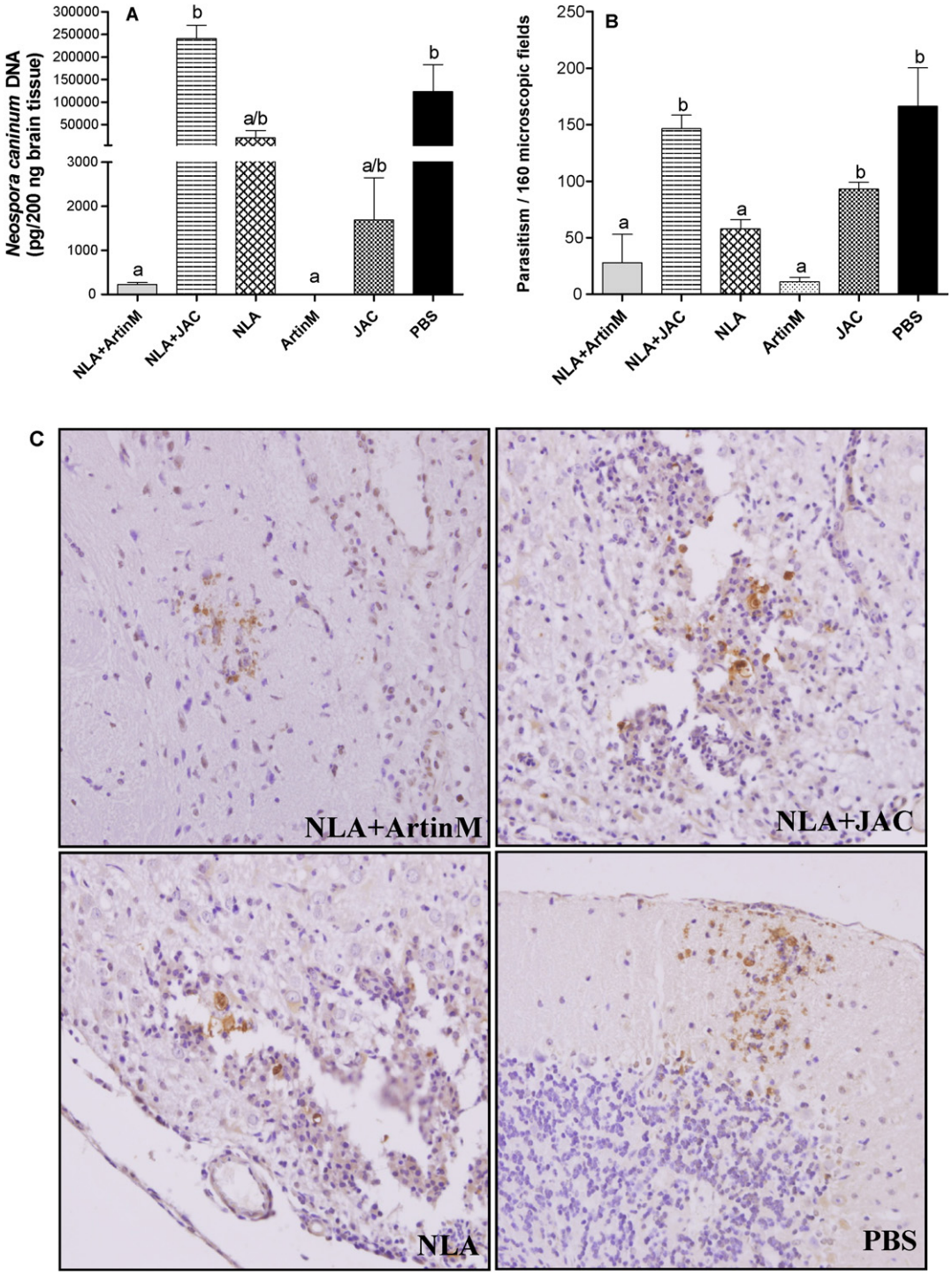

Fig. 6. Brain parasite burden after challenge with *N. caninum*. Six mouse groups (13 animals per group) were immunized with *Neospora* lysate antigen (NLA) associated with lectins from *A. integrifolia*, ArtinM (NLA + ArtinM) or Jacalin (NLA + JAC). As controls, mice were inoculated with NLA alone (antigen control), ArtinM or JAC alone (lectin controls) or PBS (infection control). Mice (10 animals per group) were challenged with Nc-1 isolate at 60 days after immunization and brain parasite load was analyzed by real-time PCR (A) and immunohistochemical assay (B) from all surviving mice after 30 days of challenge. Bars represent mean ± SEM of two independent experiments. $^{*}P < 0.05$ determined by the Kruskal–Wallis test and Dunn multiple comparison post-test. (C) Representative photomicrographs of immunohistochemical assays in brain tissues from mice of NLA + ArtinM, NLA + JAC, NLA and PBS groups, showing strongly stained *N. caninum* parasitophorous vacuoles and free tachyzoites (original magnification, ×40).

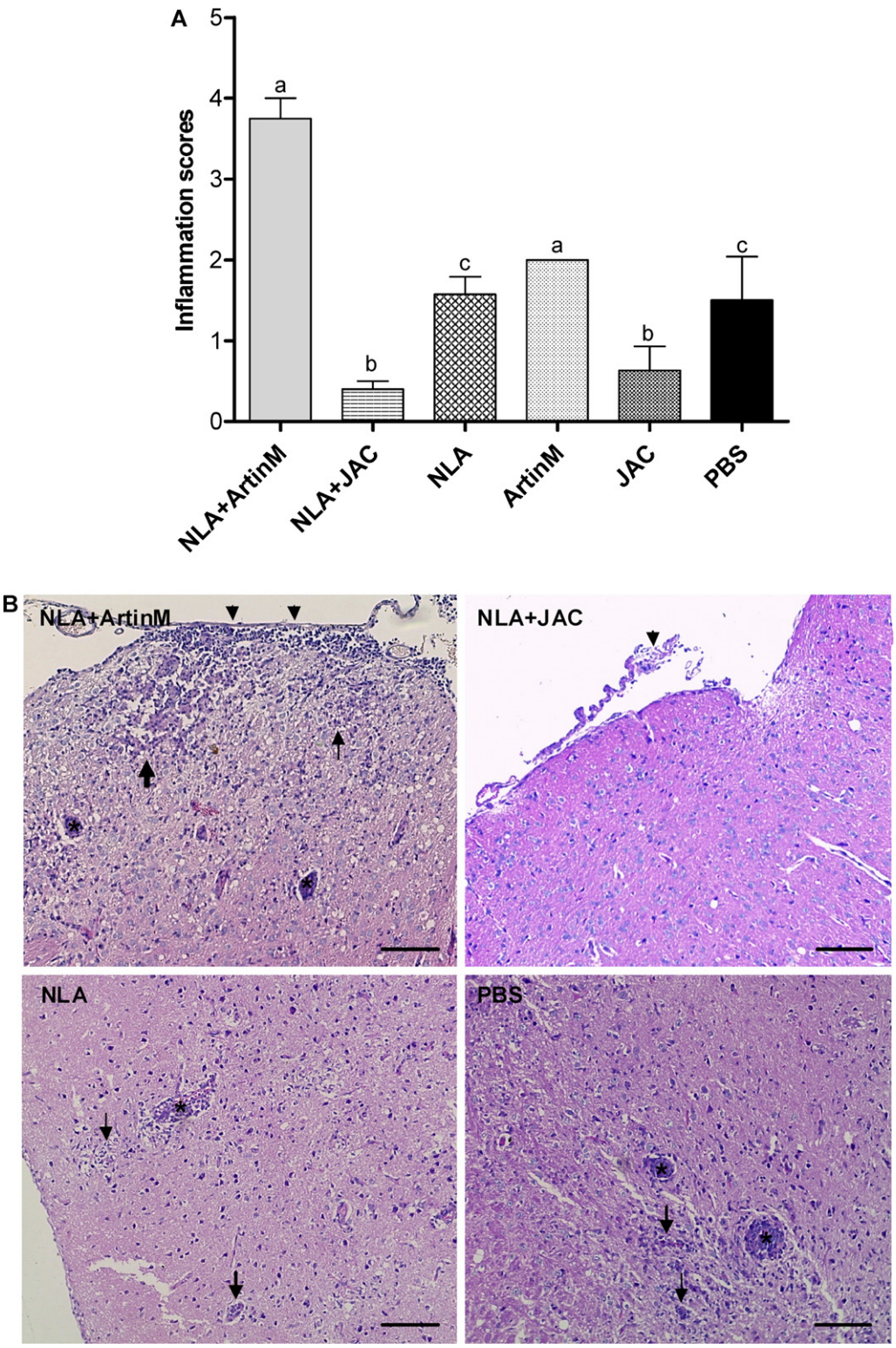

Fig. 7. Inflammation scores in brain tissues after *N. caninum* challenge. Six mouse groups (13 animals per group) were immunized with *Neospora* lysate antigen (NLA) associated with lectins from *A. integrifolia*, ArtinM (NLA + ArtinM) or Jacalin (NLA + JAC). As controls, mice were inoculated with NLA alone (antigen control), ArtinM or JAC alone (lectin controls) or PBS (infection control). Mice (10 animals per group) were challenged with Nc-1 isolate at 60 days after immunization and histological changes were analyzed. (A) Inflammatory scores in brain tissue from all surviving mice after challenge with Nc-1 isolate. Bars represent mean ± SEM of two independent experiments. $^{a-c}$Different letters indicate statistically significant differences among the groups ($P < 0.05$; ANOVA and Bonferroni multiple comparison post-test). (B) Representative photomicrographs of histological assays in brain tissues from mice of NLA + ArtinM, NLA + JAC, NLA and PBS groups. Asterisks (*) represent vascular cuffing by leukocytes, arrowheads indicate mononucleated inflammatory cell infiltrates in the meninges, and arrows indicate inflammatory cell infiltration in the parenchyma. H&E staining. Bar scale: 100 μm.

($P < 0.05$), whereas NLA and JAC groups showed similar parasite burden with no significant difference in relation to NLA + JAC and PBS groups. Brain tissue parasitism was also evaluated by immunohistochemical assay (Fig. 6B) and showed similar results to PCR data, with a lower parasitism in mice immunized with NLA + ArtinM and ArtinM, in addition to NLA alone, when compared to NLA + JAC, PBS and JAC groups ($P < 0.05$), which showed similar tissue parasitism among them. Representative photomicrographs of antigen-immunized groups and PBS group after challenge are shown in Fig. 6C, with strongly stained free parasites or within parasitophorous vacuoles.

Concerning the brain inflammation (Fig. 7A), mice immunized with NLA + ArtinM and ArtinM alone showed the highest inflammation scores in relation to all other groups ($P < 0.05$), whereas NLA + JAC and JAC groups presented the lowest inflammation scores ($P < 0.05$). The brain histopathological changes included lesions characterized by mononucleated cell infiltrates in the parenchyma, glial nodules, vascular cuffing by lymphocytes and focal mononucleated cell infiltrates in the meninges (Fig. 7B).

4. Discussion

Control of neosporosis in cattle involves three main options: (i) a yet hypothetical treatment with a parasiticide drug; (ii) a test-and-cull approach, where infected animals are identified and eliminated from the herd; and (iii) a vaccination strategy. From these options, economic analyses suggest that vaccination might be the most cost-effective approach in controlling neosporosis [17].

Previous studies have investigated live [19], gamma-irradiated [21] tachyzoites, or live tachyzoites attenuated through high passage in cell culture [18] as candidate antigens in immunization procedures. Other studies have approached immunization against *N. caninum* using recombinant proteins, such as NcSRS2 and NcSAG1 [23,27], NcSAG4 and NcGRA7 [34], GRA1, GRA2 and MIC10 [25], among others. In addition, classically known adjuvants as Freund adjuvant and ISCOMs (immune stimulating complex formulations) have been used along with native *Neospora* antigens in vaccination strategies [19]. Several native antigens have been evaluated, such as whole *Neospora* lysate antigen (NLA) [22,29,35] and excreted-secreted antigens (NcESA) [29], showing varied levels of protection of mice challenged with lethal dose of the parasite. In our previous study, we found that NLA combined with ODN-CpG adjuvant enhanced protection against *N. caninum* infection in mice, whereas immunization with NcESA resulted in a strong cellular immune response associated with high levels of IFN-γ and inflammation, rendering mice more susceptible to parasite challenge [29]. Recent studies have shown that protein vaccines with different delivery systems, such as chitosan-based nanogels (with or without mannosylated surfaces) [36] and oligomannose-coated liposomes [37], seem to be effective to control neosporosis in murine models. Therefore, in addition to the nature of antigen, the protective effect of vaccination also depends on the route of antigen, the delivery system and the type of adjuvant administered.

In this context, protein-carbohydrate recognition is essential to several intracellular processes, including the host-pathogen interaction and immune response [8]. Lectins have a potential role for this purpose, since they bind carbohydrates and could play an important task in the protection against *Leishmania* spp and *T. cruzi* parasites [14–16]. ArtinM, the D-mannose-binding lectin, is known to induce a Th1-biased immune response with production of IL-12 by macrophages [15] and induction of neutrophil activation, with release of inflammatory mediators and enhancement of their effector functions [38]. On the other hand, Jacalin, the D-galactose-binding lectin, was shown to be mitogenic for human CD4T lymphocytes [39] and, more recently, has demonstrated immunoregulatory actions as in HIV infection, where glycosylation-dependent interactions of Jacalin with CD45 on $CD4^+$ and $CD8^+$ T cells elevated TCR-mediated signaling, inducing secretion of IL-2, which thereby up-regulated T cell activation and Th1/Th2 cytokine secretion [40].

In the present study, the immunization of mice using the ArtinM lectin as an adjuvant for NLA induced the production of higher levels of specific IgG antibodies by those animals, when compared to Jacalin lectin associated with NLA or NLA alone. After the vaccination protocols, the induced immune responses revealed a considerably higher adjuvant capacity of ArtinM than Jacalin, given that the former was able to increase the immunogenicity of NLA, demonstrated by high levels of specific total IgG, IgG1 and IgG2a antibodies.

When comparing the IgG1 and IgG2a isotypes immediately before parasite challenge (60 d.a.i.) and after 30 days of challenge, levels of IgG1 were higher than those of IgG2a for all groups of animals immunized with antigen associated or not with lectins, showing a Th2-type associated humoral immune response that seems to be dependent of the antigen rather than the adjuvant. In contrast, an increased production of specific IgG2a after challenge was verified only in mice immunized with the ArtinM lectin alone, suggesting its immunomodulatory role towards a Th1-type associated humoral immune response. These findings are in agreement with our previous study using NLA or NcESA combined with ODN-CpG adjuvant that showed a considerable increment in both IgG1 and IgG2a isotypes after challenge in antigen-immunized groups, indicating that the parasite was able to induce both types of immune responses, although a Th2-type associated humoral response was more evident [29]. Interestingly, when comparing IgG2a/IgG1 ratio before and after challenge, a significantly increased IgG2a/IgG1 ratio after challenge was verified only in groups of mice immunized with ArtinM alone or associated with NLA, suggesting an attempt to increase IgG2a isotype response after parasite challenge by animals of these groups.

In contrast, the Jacalin lectin showed a lower adjuvant activity than ArtinM in immunization against *N. caninum*, but it was able to induce higher total IgG levels up to 45 d.a.i. when compared to NLA alone, although higher levels of IgG1 or similar IgG2a levels were obtained after immunization with NLA alone as compared with NLA + JAC group. The adjuvant effect of Jacalin, at the same dose (100 μg) herein employed, has been previously reported, showing increased levels of *T. cruzi*-specific antibodies in mice immunized with epimastigote forms of the parasite plus Jacalin [14]. The differential *N. caninum* tachyzoite immunostaining seen among groups in IFAT reinforces these serological findings, suggesting that the adjuvant choice can influence the magnitude of the immune response and confirming a stronger humoral immune response induced by NLA associated with ArtinM in comparison to Jacalin or NLA alone.

Cytokine production after antigenic stimulation showed that NLA plus ArtinM induced the highest levels of IFN-γ in comparison to the other groups. These results support previous data showing that ArtinM induces a great IL-12p40 production by macrophages and IFN-γ by spleen cells, switching from the type 2 to type 1 cell-mediated immunity against *Leishmania major* antigens and resulting in resistance to infection [15]. Another study evaluating the potential of the ArtinM lectin in immunization against *Leishmania amazonensis* infection showed that the combination of ArtinM with soluble *Leishmania* antigen (SLA) also induced IFN-γ production [16]. When analyzing IL-10 production after antigen stimulation, NLA + ArtinM and NLA groups exhibited higher IL-10 levels than the other groups. Interestingly, IL-10 levels produced by spleen cells after antigen stimulation were even higher than those

produced after mitogen stimulation, reinforcing the role of the NLA antigen in inducing an anti-inflammatory or immunoregulatory response. When the IFN-γ/IL-10 ratio was analyzed, however, it was observed that NLA + ArtinM and ArtinM groups presented the highest IFN-γ/IL-10 ratio, suggesting that the lectin adjuvant, but not the antigen, is able to modulate the cytokine production, leading to a Th1 type-biased pro-inflammatory immune response that is considered protective against *N. caninum*. On the other hand, a non exacerbated Th1 immune response profile seems to be more appropriate to control neosporosis, since our previous study showed that vaccination with NcESA alone or combined with ODN-CpG adjuvant resulted in a strong cellular immune response associated with high levels of IFN-γ and inflammation, rendering mice more susceptible to parasite challenge [29]. Also, immunization of BALB/c mice with soluble *N. caninum* tachyzoite antigens entrapped in nonionic surfactant vesicles or administered with Freund's adjuvant had clinical neurological disease and increased numbers of brain lesions compared to groups of mice inoculated with adjuvants alone or non-immunized controls, following virulent parasite challenge [41]. These findings were associated with increased IL-4 secretion and IL-4/IFN-γ ratio *in vitro* as well as increased IgG1/IgG2a ratio in vivo, showing that the induction of a type 2 immune response is not protective to neosporosis [41].

Although the best way to infer about a Th1 or Th2 biased immune response should be the IFN-γ/IL-4 ratio determination, we have demonstrated in our previous study [29] that IL-4 was consistently undetectable in supernatants from C57BL/6 mouse spleen cell cultures, even using high sensitivity commercially available kits with a limit of detection of 15 pg/ml. Thus, the IFN-gamma/IL-10 ratio was adopted in an attempt to verify the balance between pro-inflammatory and anti-inflammatory cytokines. As we observed that the highest IFN-gamma/IL-10 ratio was found for the NLA + ArtinM group followed by the ArtinM group in relation to the remaining groups, these data could indicate a profile of Th1-biased pro-inflammatory immune response, supporting the role of ArtinM as a strong inducer of Th1-type immune responses, as demonstrated in other infection models [15,16].

In the present study, a protective pattern of Th1-biased pro-inflammatory immune response can have influenced the survival of the animals after parasite challenge, given that mice immunized with NLA + ArtinM presented the greatest survival and the lowest brain parasite load, indicating that increased IgG2a levels before challenge, higher IgG2a/IgG1 ratio after challenge and higher IFN-γ/IL-10 ratio after immunization can be associated with protection against infection. However, the mouse groups that received ArtinM with or without antigen presented the highest morbidity scores and weight changes from baseline. It is noteworthy that these parameters were more remarkable during the acute phase of infection (from 7 to 12 days after challenge), being the higher rates of body weight losses coincident with the peak of morbidity scores. Afterward both parameters showed a tendency to return to the baseline, contributing to the higher survival rate observed mainly in the NLA + ArtinM group. Also, inflammation scores in brain tissues after parasite challenge predominated in mice immunized with NLA + ArtinM and ArtinM alone. These findings are likely associated with the enhanced IFN-γ/IL-10 and IgG2a/IgG1 ratios after parasite challenge observed in these animals, reflecting in a Th1-type biased pro-inflammatory immune response induced in the acute phase of the infection.

It is well known the role of T CD4+ cells and mostly IFN-γ to control *N. caninum* infection [6]. On the other hand, the induction of a type 2 immune response associated with a pattern of anti-inflammatory response is not protective to neosporois [41]. Therefore, we believe that a non-exacerbated pro-inflammatory immune response is associated with the host resistance to parasite infection and consequently the progression to the asymptomatic chronic phase of neosporosis. Accordingly, in our experimental design, the induction of a pro-inflammatory immune response by ArtinM associated with NLA showed to be beneficial rather than deleterious to the host to control neosporosis. A previous study also showed that the combination of ArtinM with soluble *Leishmania* antigen (SLA) induced IFN-γ production, thus reducing the parasite load, but without decreasing the lesion size [16].

Interestingly, in the present study, the survival curves showed deaths occurring earlier than our previous report [29], although we have used the same mouse lineage and the same tachyzoite number (2×10^7 tachyzoites/mouse) for challenge. An explanation for these findings is likely because we employed in the present study a *N. caninum* isolate from lower passage than that used in our previous study. Accordingly, it is known that long-term passage of tachyzoites in tissue culture can attenuate virulence of *N. caninum* in vivo [32].

On the other hand, mice immunized with NLA + JAC or NLA alone presented an anti-inflammatory or immunoregulatory profile, leading to higher parasite burden, suggesting that the immune response induced in these groups was not effective. In contrast, a previous study evaluating the adjuvant effect of Jacalin associated with epimastigote forms of *T. cruzi* showed that the parasite load of mice immunized was reduced after challenge with trypomastigotes in relation to the group immunized with parasite alone [14].

Surprisingly, mice immunized with the ArtinM lectin alone showed the lowest brain parasite load compared to the other groups, although with no significant difference to the NLA + ArtinM group. This finding associated with enhanced IgG2a/IgG1 ratio after parasite challenge and increased IFN-γ/IL-10 ratio observed in ArtinM group, may indicate that the immune stimulating effect of the ArtinM lectin itself may be a good target for therapies and it can stimulate an innate immune response dependent of the Toll-like 2 receptor for production of IL-12. In this context, studies have demonstrated that treatment with ArtinM was able to recruit and activate innate immune cells, especially the neutrophils, inducing a potent immunostimulatory effect [38] as well as to confer protection against *Paracoccidioides brasiliensis* [42]. The mechanism of action of ArtinM in these studies was shown to be dependent of the Toll-like 2 receptor for production of IL-12. More recently, the prophylactic administration of ArtinM in both native and recombinant forms showed protection against *P. brasiliensis*, with reduction of the fungal load and the incidence of granuloma, associated with increased levels of IL-12, IFN-γ, TNF-α and NO, inducing protective Th1-type immune response [43].

Previous studies showed that the particular delivery vehicle may bias the immune response towards a more active response, and innate responses are likely important for determining the protective effects in these models, stimulating the parasite-specific Th1 immune response and antibody responses. These data reinforce that protein–carbohydrate binding is important in the immune response against *N. caninum*. In the present study, the mannose-binding is somehow necessary for this effect, since the mannose-binding lectin ArtinM was a better adjuvant than the galactose-binding lectin Jacalin in immunization against neosporosis.

Altogether, it can be concluded that the ArtinM lectin promotes resistance against *N. caninum* in immunized mice, through the induction of Th1-biased pro-inflammatory immune response, constituting a potential adjuvant candidate for vaccine formulations against neosporosis and should be approached in subsequent investigations in congenital infection models. In addition, considering that the current vaccination strategies against neosporosis in the field are demonstrating low efficacy, as they result in partial protection, our findings may constitute an inexpensive and viable method for herd vaccination.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.09.136.

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